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Multiple heart-cutting two dimensional liquid chromatography and isotope dilution tandem mass spectrometry for the absolute quantification of proteins in human serum

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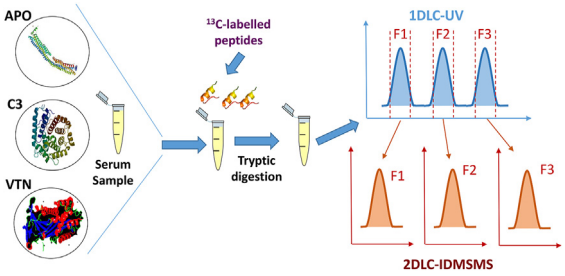
HIGHLIGHTS

- Multiple heart cutting and isotope dilution for peptides enrichment and quantification.
- Direct analysis of tryptic digests of serum for quantification of glaucoma biomarkers.
- Use of mobile phases not compatible with ESI while minimizing ionization suppression.
- Satisfactory sample throughput and extension to a higher number of proteins.

GRAPHICAL ABSTRACT



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ABSTRACT

We evaluate here the combination of two-dimensional liquid chromatography (2D-LC) in the multiple heart cutting mode and isotope dilution tandem mass spectrometry for the direct analysis of tryptic digests of serum samples. As a proof of concept, we attempt the quantification of proteotypic peptides of Apolipoprotein AIV (APOA4), Complement C3 (C3) and Vitronectin (VTN) which have been previously identified as potential candidate biomarkers of glaucoma. Using this 2D-LC strategy, analyte enrichment steps are avoided and the sample preparation involved after enzymatic digestion amounted to a simple centrifugation, evaporation of the supernatant and reconstitution in the 1D mobile phase. A mobile phase not compatible with the ESI source (10 mM KH_2PO_4 at pH 2.7) was used in the first dimension as it provided a satisfactory chromatographic resolution of the peptides and a high buffering capacity avoiding changes in retention times when analyzing complex matrices like human serum. We also demonstrate that using coeluting labelled analogues of the target peptides, protein concentrations were not affected by slight retention time shifts affecting the amount of target peptides transferred to the second dimension. Satisfactory results were obtained when analyzing fortified serum samples (recoveries from 98 to 113%). Precisions in the range of 1–9% RSD were obtained when replicating the analysis of a pooled serum sample. The comparative analysis of serum samples from n = 94 control subjects and n = 91 patients diagnosed with primary open-angle glaucoma did not show significant

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differences in the APOA4, VTN and C3 concentrations in contrast with previous studies using immunoassays.

1. Introduction

Clinical applications based on Mass spectrometry (MS) are continuously expanding in the laboratory medicine [1]. MS typically provides greater selectivity and sensitivity than spectrophotometric and immunogenic assays and is considered the most powerful technique for the discovery of new clinical biomarkers [2]. Among the available mass spectrometric techniques LC-ESI-MSMS is the most viable and cost-effective analytical platform in the clinical laboratory [3,4]. However, accurate and precise quantification of trace amounts of clinical biomarkers in human samples by LC-ESI-MS/MS is still challenging [5]. Ionization suppression effects in ESI due to matrix effects significantly affect the instrumental sensitivity and the accuracy and precision of the results [6,7]. Strategies to minimize matrix effects in ESI include specific dedicated clean-up protocols, post-column infusion, matrix dilution and standardization based on the use of standard additions, external matrix matched standards or analogue-structure internal standards [7]. The use of isotopically labelled analogues as internal standards to apply isotope dilution mass spectrometry (IDMS) is widely regarded as the most efficient standardization strategy [8]. Coelution of analyte and labelled analogue after chromatography is required for an adequate correction of matrix effects. However, even when using adequate labelled analogues, matrix constituents in complex samples can lead to serious signal suppression or spectral interferences and hence, specific dedicated sample purification approaches are required.

Clinical proteomics investigate the changes in the concentrations of proteins and peptides in tissues and body fluids in response to diseases or external stimulation [9]. In clinical laboratories most of the methods applied for the quantification of proteins or peptides are based on immunoassays. However, targeted MS are becoming an alternative to solve the inherent flaws associated to immunoassays [10]. Most of MS workflows typically involve an optimized protein digestion using proteases to cleave the protein into smaller peptides and the addition of an isotopically labelled analogue of a proteotypic peptide that will be surrogate for the protein measurement [11–13]. When quantifying low abundance proteins in complex samples such as serum or plasma, the application of protein or peptide enrichment steps are required to avoid interferences or ion suppression effects [14]. Analyte enrichment steps in clinical proteomics can be based on high abundance proteins precipitation, size exclusion, dialysis, solid-phase extraction, electrophoresis or immunoaffinity strategies [15]. Strategies based on the use of antibodies to enrich proteins before digestion or specific peptides after digestion have proved to be the most effective in improving method detection limits [16,17].

Two-dimensional liquid chromatography (2D-LC) enhances the separation power compared to conventional LC particularly in complex samples [18]. 2D-LC modes include comprehensive (LCxLC) and heart-cutting (LC-LC) analysis. Multiple heart-cutting (MHC) is an intermediate mode in which a predefined number of fractions from the first dimension (1D) chromatogram are subjected to the second dimension [19]. Using the MHC mode several analytes can be subjected to two different separation mechanisms so that a purification of the sample is achieved while increasing the chromatographic resolution between analytes and interfering matrix compounds [20,21]. In addition, this strategy is particularly useful when using mobile phases in the first dimension which are not compatible with the ESI source due to the presence of non-volatile salts or organic modifiers [22].

Automated multiple heart-cutting devices have been coupled to mass spectrometry (MHC-MS) mostly for qualitative analysis such as characterization of antibodies [23], plant species [24] or synthetic oligonucleotides [25]. Quantitative applications of LC-LC and IDMS been restricted so far to the quantification of small organic molecules [26–28], nucleotides [29] or aflatoxins [30,31]. Peptide quantitation applying IDMS and on-line two dimensional reversed phase methods have been reported by trapping and elution before the second dimension [32]. However, MHC-MS has not been applied so far to the absolute quantification of proteins or peptides by IDMS. This work evaluates for the first time, the combination of MHC and isotope dilution tandem mass spectrometry as a peptide enrichment and quantitative strategy in bottom-up proteomics. In this way, traditional analyte enrichment steps are avoided and the digested serum can be directly analyzed after a centrifugation and reconstitution in the 1D mobile phase. As a proof of concept, we present the direct analysis of tryptic digests from serum samples to quantify proteotypic peptides of the proteins Apolipoprotein AIV (APOA4), Complement C3 (C3) and Vitronectin (VTN) which have been previously identified as potential biomarkers of glaucoma [33].

2. Experimental

2.1. Instrumentation

An Agilent 1290 Infinity 2D-LC system coupled to a triple quadrupole mass spectrometer Agilent 6460 equipped with an electrospray source with a jet stream was used throughout this work. The 2D-LC system was controlled by OpenLab CDS Chemstation and the triple quadrupole by MassHunter Acquisition software (Agilent Technologies). The first dimension incorporated a 1290 Infinity binary pump connected to an autosampler, thermostated column compartment and a 1260 Infinity variable wavelength detector with a 10 mm flow cell. The two dimensions were interconnected by a 2-pos/4-port duo valve to which two distinct selector valves including six 40 μL sampling loops were coupled.

A vortex mixer (FB 15024, Fisher Scientific, Hampton, NH, USA) was used for the homogenization of samples and working solutions. All standard solutions and mixtures were prepared gravimetrically using an analytical balance model MS205DU (Mettler Toledo, Zurich, Switzerland). Ultra-pure water was obtained from a Purelab Flex 3 water purification system (Elga Labwater, Lane End, UK). A centrifugal vacuum concentrator (Genevac, Suffolk, UK) was used to remove water and organic solvents.

2.2. Reagents and materials

All solvents and reagents were of analytical reagent grade. Bovine trypsin (treated with L-1-tosylamide-2-phenylethyl chloromethyl ketone) was used for the sample preparation of serum samples, trypsin from porcine pancreas (20 μg/ampule, proteomics grade), iodoacetamide (alkylating reagent, 98%, IAA) was used for

the hydrolysis of protein standards, dithiothreitol (reductant agent, >99%, DTT), urea (>99.5%), ammonium acetate (>99%), potassium dihydrogen phosphate (99.5%), potassium chloride (>99%), ammonia (>99%), formic acid (>98%) and trifluoroacetic acid (>99%, TFA), were purchased from Sigma-Aldrich (St. Louis, MO, USA). Trypsin solutions were obtained by dissolving the enzyme in acetic acid (50 mM). DTT and IAA solutions were prepared by dissolving these reagents in ultra-pure water. Recombinant human VTN, C3 and APOA4 were purchased from Sigma-Aldrich.

2.2.1. Peptide standards

Two different proteotypic peptides for each of the three target proteins APOA4, VTN and C3 were synthesized in house using a microwave peptide synthesizer Liberty Blue from CEM Corporation (Matthews, NC, USA). Both natural abundance and ^{13}C labelled analogues of each proteotypic peptides were synthesized. The purification of the synthesized crudes was carried out by semi-preparative liquid chromatography using an Agilent 1260 Infinity HPLC system (Agilent Technologies) equipped with an analytical-scale fraction collector and a variable wavelength detector. The column was an Aeris Peptide XB-C18 5um 100A, 250 × 4.6 mm from Phenomenex (Torrance, USA). Purified peptides were lyophilized using a Heto PowerDry LL3000 Freeze Dryer from Thermo Fisher Scientific (Waltham, MA USA). The isotopic enrichment of the labelled analogues was calculated as described in a previous publication [34]. Table 1 shows the sequence, labelling position and isotopic enrichment of the synthesized labelled peptides. Labelled peptides containing only 1 or 2 ^{13}C atoms ($^{13}C_2$-Glycine or $^{13}C_1$-Valine) were selected in this work.

2.2.2. Study subjects and serum sample collection

Patients with primary open-angle glaucoma (POAG, n = 91) and control subjects (n = 94) were recruited and screened at the Institute of Ophthalmology Fernandez-Vega (Oviedo, Spain) and classified according to their age and gender. All subjects included in this study signed an informed consent and had complete ophthalmologic examinations, including slit-lamp biomicroscopy, funduscopy, gonioscopy, perimetric field loss, intraocular pressure and analysis of nervous fibers. The diagnostic criteria for POAG included the presence of characteristic optic-disc damage (e.g., vertical cup-to-disc ratio >0.3, thin or notched neuroretinal rim, or disc hemorrhage) with the corresponding characteristic changes in the visual field and the presence of an open anterior chamber angle (Shaffer grade III or IV). No subjects involved in this study presented any other relevant ocular pathology, such as retinopathies or maculopathies. Control subjects were selected from patients undergoing cataract surgery and absence of glaucoma. The study adheres to the tenets of the Declaration of Helsinki on Biomedical Research Involving Human Subjects, and full ethical approval was obtained from the Clinical Research Ethics Committee (Code 102/13) at Principality of Asturias (Oviedo, Spain). Demographic characteristics of the recruited patients and control individuals are shown in Table S1. Peripheral blood samples were drawn from each participant collected in 5 mL Z Serum Sep Clot Activator tubes) coated with microscopic silica particles (Vacuette, Madrid, Spain). The sera were separated from clotting factors and blood cells by centrifugation (1,800 g for 18 min at 4 °C) and stored at −80 °C until use. A total number of 185 serum samples were analyzed.

2.3. Procedures

2.3.1. Sample treatment and enzymatic hydrolysis

For the enzymatic digestion of proteins APOA4, VTN and C3 an amount of 0.07 g of solid urea was added to 0.050 g of human serum to obtain a concentration of 6 M. Then, the Eppendorf was vortexed for 30 s and 0.20 mL of ammonium acetate buffer (pH 8 and 100 mM) was added to dilute the sample 1:1. Reduction of cysteine residues was carried out by mechanical shaking for 30 min at 37 °C after the addition of 0.020 mL of 200 mM DTT. Then, the alkylation of cysteine residues was carried out by adding 0.023 mL of 600 mM IAA and developing the reaction for 30 min in the dark and at room temperature. The excess of IAA was quenched by the addition of 0.025 mL of 600 mM DTT. Before proteolysis, the concentration of urea was reduced to 2 M with the ammonium acetate buffer. Then, a gravimetrically controlled amount of the ^{13}C-labelled peptides was added and the enzymatic digestion was carried out adding the amount of trypsin required to yield a 1:10 enzyme to substrate ratio (w/w) for 22 h at pH 8 and 37 °C. The enzymatic reaction was stopped by the addition of 30 μL of TFA at 20% (v/v) to obtain a pH < 2. Finally, the sample was centrifuged at 14000 g for 15 min and the supernatant was taken and evaporated to dryness.

2.3.2. Chromatographic separation of the samples by MHC-LC-ESI-MSMS

The 1D-column was a ZORBAX 300 Å SB-C18 Rapid resolution HD 2.1 × 100 mm 1.8 μm (Agilent Technologies) held at 30 °C. Mobile Phases A and B were 10 mM KH_2PO_4 H_2O at pH 2,7 and a 30:70 mixture of water and acetonitrile, respectively. A sample volume of 4 μL was injected and the flow rate was set at 0.4 mL/min. Prior to injection, the needle underwent a wash cycle for 6 s with a mixture of 25:25:25:25 acetonitrile/methanol/water/isopropanol. The chromatographic method of the first dimension held the initial mobile phase composition (2.85% B) constant for 3 min. Then, a linear gradient to 50.28% B up to 11 min was applied and kept for 6 min. Finally, a gradient to 100% B up to 18 min was kept for 15 min. The initial mobile phase composition was applied after 1 min and equilibrated with the column for 7 min until the 2D separations were completed. The second dimension incorporated also a 1290 Infinity binary pump (Agilent Technologies). Taking into account the retention time of the target peptides VTN 1, APO 1, C3 1, C3 2 and VTN 2, fractions of 40 μL of the mobile phase of the first dimension were stored in the sampling loops at 6.00, 6.64, 7.73, 8.33 and 11.01 min, respectively. Once the last peptide was stored (VTN 2), they were transferred to the second dimension in reverse order. The 2D-column was a 100 Å Aeris Peptide XB-C18 2.1 × 100 mm 1,7 μm (Phenomenex) and was also held at 30 °C. Mobile phases A and B were 0.1% formic acid in water and acetonitrile, respectively. Gradients went from 2%B to 80%B in 4 min and

Table 1
Amino acid sequence, labelling position and isotopic enrichment of the minimally labelled proteotypic peptides employed in this work.

Protein	Peptide	Peptide sequence	Peptide sequence for labelled peptide	Isotopic enrichment (%)
Vitronectin	VTN 1	AVRPGYPK	AVRP($^{13}C_2$-G)YPK	99,26 ± 0,01%
Vitronectin	VTN 2	DVWGIEGPIDAAFTR	DVWGIE($^{13}C_2$-G)PIDAAFTR	99,06 ± 0,02%
Apolipoprotein AIV	APO 1	LAPLAEDVR	LAPLAED($^{13}C_1$-V)R	99,30 ± 0,06%
Apolipoprotein AIV	APO 2	LLPHANEVSQK	LLPHANE($^{13}C_1$-V)SQK	98,5 ± 0,2%
Complement C3	C3 1	VVLVAVDK	VVL($^{13}C_1$-V)AVDK	99,15 ± 0,06
Complement C3	C3 2	IWDVVEK	IWD($^{13}C_1$-V)VEK	98,8 ± 0,2%

the flow rate was established at 0.4 mL/min. A post-analysis time of 2 min was applied for column equilibration. Since the last peptide eluted from the 1D Column at 12 min the overall analysis stop time was set to 42 min to ensure the injection of all heart-cuts in the second dimension.

2.3.3. Measurement of the isotopic composition of the target peptides in serum samples by MHC-LC-ESI-MS/MS

The 2D-LC system was coupled to a triple quadrupole mass spectrometer equipped with an electrospray source working in positive ion mode. The ionization source working conditions are given in Table S2 of the Supporting Information. For all peptides, doubly charged ions $[M+2H]^{2+}$ were selected as precursor ions and all the samples were measured using the Selected Reaction Monitoring mode (SRM). Fragmentation of the precursor ions by CID were carried out with nitrogen as collision gas using the experimental conditions described in Table S3 of the Supporting Information. The isotope distribution of the in-cell fragment ions measured by SRM were compared with the theoretical values obtained by the SRM dedicated software such as IsoPatrn© [35] with satisfactory results. As described previously [36], direct quantification of the target peptides is enabled using the molar fractions of the analyte and labelled analogue obtained from the measurement of the isotopic composition of the samples. This is facilitated by the use of Mass Overlapping Peptides (MOPs) labelled with 1 or 2 ^{13}C atoms [36].

Measurement of the samples by 1D-LC-ESI-MSMS during the optimization of the enzymatic digestion step with trypsin.

Recombinant human VTN, APOA4 and C3 were denaturalized with DTT and alkylation of cysteine residues was carried out with IAA. Then, the six ^{13}C-labelled peptides were added and the enzymatic digestion was carried out adding the amount of trypsin required to yield a 1:16 enzyme to substrate ratio (w/w) at pH 8 and 37 °C. Aliquots of 0.03 mL were taken at 0, 15, 30, 45, 60, 90, 120, 180, 240, 300, 360, 430, 540, 660, 720, 1305, 1365 and 1485 min. To stop the reaction in each aliquot 0.01 mL of TFA (20% v/v) was added. After evaporation the aliquots were dissolved in 0.015 mL of 0.1% formic acid and analyzed by LC-MSMS. The aliquots were measured by 1D-LC-ESI-MSMS using a chromatographic column Aeris Peptide XB-C18 (2,1 × 100 mm, 1,7 μm) from Phenomenex. A sample volume of 15 μL was injected and the flow rate was set at 0.4 mL/min. Mobile Phases A and B were 0.1% formic acid in ultrapure water and acetonitrile respectively. The chromatographic method held the initial mobile phase composition (2% B) constant for 3 min. Then, a linear gradient to 12% B up to 7 min was applied. Then. a second gradient to 13%B up to 9 min and a third gradient to 50%B up to 13 min were applied. Finally, a linear gradient to 80% B up to 17 min was applied and the initial mobile phase composition was applied after 1 min. The SRM transitions were those detailed in the previous section for 2D-LC-MS/MS measurements.

3. Results and disscussion

3.1. Optimization of the enzymatic digestion

The absolute quantification of proteins using isotopically labelled proteotypic peptides requires their complete digestion to release the endogenous peptides. Also, if the labelled peptide is added at the beginning of the process, it must be stable during the enzymatic digestion so that both analyte and labelled analogue can be completely mixed in a homogenous liquid phase. The time required for a complete digestion must be specifically optimized for each target protein. Fig. S1 shows the concentration of the endogenous proteotypic peptides obtained for APOA4, VTN and C3. For APO, Fig. S1A shows that the same peptide concentration is obtained for both fragments of peptide APO 1 and that a constant peptide concentration value is obtained after 90 min. However, for peptide APO 2 (LAPLAEDVR), the fragment y_5^+ shows a rapid increase after 120 min and a significant variation in the concentration results. This is not the case for fragment y_7^+ so this could be attributed to the presence of spectral interferences rather than a poor stability of the endogenous or exogenous APO 2 peptide in the digestion medium. According to this, peptide APO 2 had to be rejected as labelled analogue in further experiments.

For the case of VTN a similar behavior in all the measured fragments of both proteotypic peptides was observed during the digestion process. According to the results shown in Fig. S1B the complete release of both peptides from the protein chain was observed after 1300 min. Concerning C3, a similar evolution was observed for both peptides during the digestion process. Also both fragments of each peptide provide the same peptide concentration. However, a significantly longer digestion time is required to release the proteotypic peptides from the protein. According to Fig. S1C the digestion time should be higher than 22 h. Thus, a digestion time of 24 h was selected for further experiments to ensure a complete digestion of C3.

3.2. Recovery studies of the proteotypic peptides in the absence of sample matrix by 1D-LC-MSMS

Finding a medium in which all the peptides standards (natural abundance and isotopically labelled) are completely dissolved is required for a proper validation of the methodology. Hoofnagle et al. [37] recommended 5%–30% acetonitrile with 0.1% formic acid depending on the hydrophobicity of the peptides. In our case we found that 15% acetonitrile with 0.1% formic acid was a suitable medium to obtain homogeneous solutions for all our target peptides. First, the labelled peptides were characterized in terms of isotopic enrichment as described in a previous publication [34]. Secondly, the concentration of labelled peptides was determined by reverse IDMS experiments using natural abundance peptides of known concentration. The precision obtained in the reverse IDMS experiments were in all cases lower than 1.4% expressed as the RSD (%) of n = 3 independent replicates for each labelled peptide demonstrating a satisfactory homogeneity of the peptides in the selected medium.

Then, recovery studies in the absence of matrix were carried out for the five peptides. A volume of 50 μL of a solution containing the five target peptides in a concentration of approximately 5 μg g^{-1} was considered as a matrix-free sample. The denaturalization protocol was applied as described in the Experimental section. Then, a volume of 50 μL of a solution containing a mixture of the labelled analogues in a concentration of approximately 5 μg g^{-1} was added and the enzymatic digestion was started. The isotopic composition of the samples was measured by 1D-LC-MSMS. The results obtained for the five peptides are given in Table S4. Recovery values between 97% and 107% were obtained for all fragments and peptides. The precision obtained between three independent 1D-LC-MSMS injections ranged from 0.03% to 8% expressed as RSD (%) and the precision obtained between three independent replicates of the same sample ranged from 0.2 to 4.7% (%RSD).

3.3. Optimization of the MHC-HPLC-MSMS measurement of the proteotypic peptides

The coupling of 2D-LC in the multiple heart cutting mode to an ESI source provides two potential advantages. First, the use of mobile phases not compatible with ESI ionization but providing improvements in the chromatographic resolution and retention time stability. Secondly, a minimization of the ionization

suppression effects due to the lower introduction of sample matrix into the ESI source. Taking into account both advantages, we aimed at developing a direct analysis of serum samples without the application of time-consuming sample purification steps required in previous publications [36]. Besides the enzymatic digestion, the sample preparation involved a simple centrifugation, evaporation of the supernatant and reconstitution in 15% acetonitrile with 0.1% formic acid.

3.3.1. Selection of the 1D mobile phase

When optimizing the MHC strategy the first step is the selection of chromatographic conditions in the first dimension that provide a complete separation of the analytes with a satisfactory reproducibility of the retention times. Two different chromatographic conditions were tested. The first was based in the use of TFA instead of formic acid to improve peak shape and chromatographic resolution of the peptides. The second was based on the use of 10 mM KH_2PO_4 at pH 2.7 as aqueous mobile phase. Both chromatographic separations were evaluated coupling the first dimension to a UV-VIS detector working at $\lambda = 220$ nm. Fig. S2 shows the separation of a mixture of the five target peptides obtained with both chromatographic conditions. As can be observed, both conditions provided baseline resolution of all target peptides so that a fraction for each peptide could be transferred without coelution of the other target peptides to the second dimension. However, the use of 10 mM KH_2PO_4 at pH 2.7 was finally selected for the analysis of serum samples as it provides a higher buffering capacity than TFA. This is particularly important to avoid changes in retention times when analyzing complex matrices like human serum.

3.3.2. Optimization of the time window for the transfer of the 1D fractions

Fig. 1 shows the comparison of a HPLC-UV chromatogram of a 5 μg g^{-1} standard mixture of the five target peptides with a tryptic digest of a pooled serum sample. As can be observed, when analyzing serum samples, the lack of selectivity of the UV-VIS detector does not allow the identification of the target retention times due to the presence of matrix constituents and the low concentration of the peptides in the sample. Therefore, the time windows of the 1D fractions must be optimized before each measurement session injecting a fortified serum sample.

Also, it is important to check the influence of retention time shifts on the peptide concentrations when injecting real samples. The MHC mode allows a high resolution sampling of each chromatographic peak to determine and confirm the retention time of each peptide in the presence of sample matrix. This strategy allows the collection of several 40 μL fractions within the chromatographic peak profile of the first dimension and transfer each fraction to the second dimension. In this way it was possible to elucidate which 40 μL fraction contains the highest amount of peptide and also if chromatographic isotopic effects between the natural abundance and labelled peptides were present. Using the high resolution sample mode, we could also evaluate if the proposed strategy was able to provide accurate concentrations as long as a detectable amount of peptides were transferred to the second dimension regardless of the total peptide concentration in each fraction. First, we analyzed a standard mixture containing a known concentration (approximately 5 μg g^{-1}) of each peptide by IDMS adding ^{13}C minimally labelled peptides. Secondly a pooled serum sample was analyzed following the optimized sample preparation protocol.

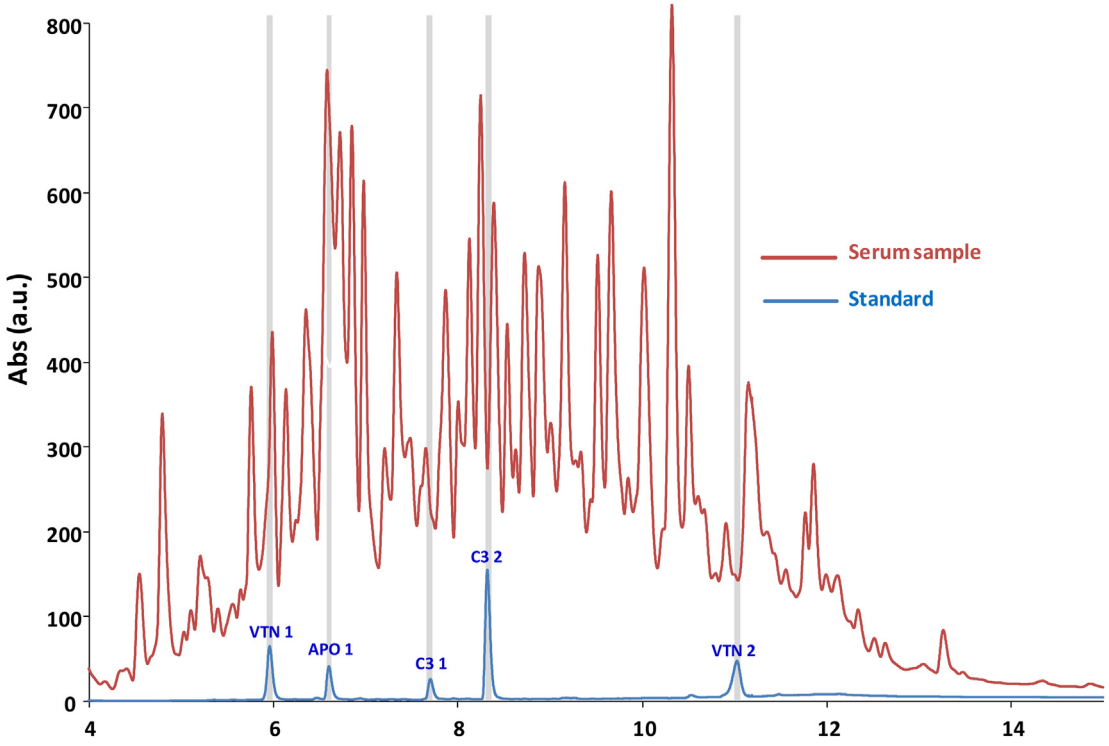

Fig. 1. Comparison of a HPLC-UV chromatogram ($\lambda = 220$ nm) of a 5 μg g^{-1} standard mixture of the peptides VTN 1, APO 1, C3 1 C3 2 and VTN 2 (blue chromatogram) with a pooled serum sample (red chromatogram). The grey lines indicate the time window of the fractions transferred to the second dimension. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Three different fractions of 40 μL were transferred to the second dimension for each peptide and the concentration of the peptides in the sample quantified in each fraction. The concentrations of the sample obtained for each fraction are given in Table S5. Also, Fig. 2 shows, the time window of each fraction and the associated concentration obtained in each fraction. As can be observed, although the second fraction transfer the highest amount of peptide to the second dimension, similar concentration results were obtained for all peptides in the three fractions. These results show that, due to the coelution of the target peptide and its minimally ^{13}C-labelled analogue, the accuracy and precision of the proposed methodology is not affected by slight retention time shifts as long as detectable amounts of the peptides are transferred to the second dimension. Fig. 3 shows both 1D-LC-UV and 2D-LC-ESI-MSMS chromatograms of a serum sample analyzed under the optimized conditions. As can be observed, when storing heart-cuts in a deck, a flush gradient is performed before analysis of the heart-cuts stored in this deck and the second-dimension analysis of stored heart-cuts is done in reversed storage order.

3.3.3. Optimization of the injection volume

The injection volume was also optimized in order to increase the sensitivity for each peptide without enhancing ionization suppression due to the sample matrix. To do so, we analyzed a pooled serum sample at different injection volumes from 1 to 5 μL. Fig. S3, shows that the sensitivity of the ESI-MS/MS system increased with the injection volume for all target peptides despite the absence of a purification step of the digested serum sample.

3.4. Recovery studies in human serum by MHC-HPLC-MS/MS

A pooled serum was selected and the endogenous concentration of the five target peptides was determined analyzing n = 3 independent replicates. The average protein concentrations and the associated standard deviation obtained from the measurement of the isotopic distribution of two different molecular fragment ions for each proteotypic peptide are shown Table S6. The RSD (%) obtained from three independent injections of the same replicate ranged from 0.2 to 6.4% whereas the RSD (%) obtained from n = 3 independent aliquots of the serum sample ranged from 0.8 to 9.6%. Then, recovery studies in the pooled sample were carried out adding to 0.05 mL of the pooled serum a known amount of the natural abundance peptides to yield an approximate peptide concentration of 30–35 μg g^{-1} in the sample. After the denaturalization step, a known amount of labelled peptides was added to yield a similar concentration than that of the hypothetical concentration of natural abundance peptides after proteolysis. Then, the digestion process was started and the samples were measured by 2D-LC-MS/MS as described in the Experimental section. The results obtained for the five peptides are given in Table 2. For peptide APO 1,

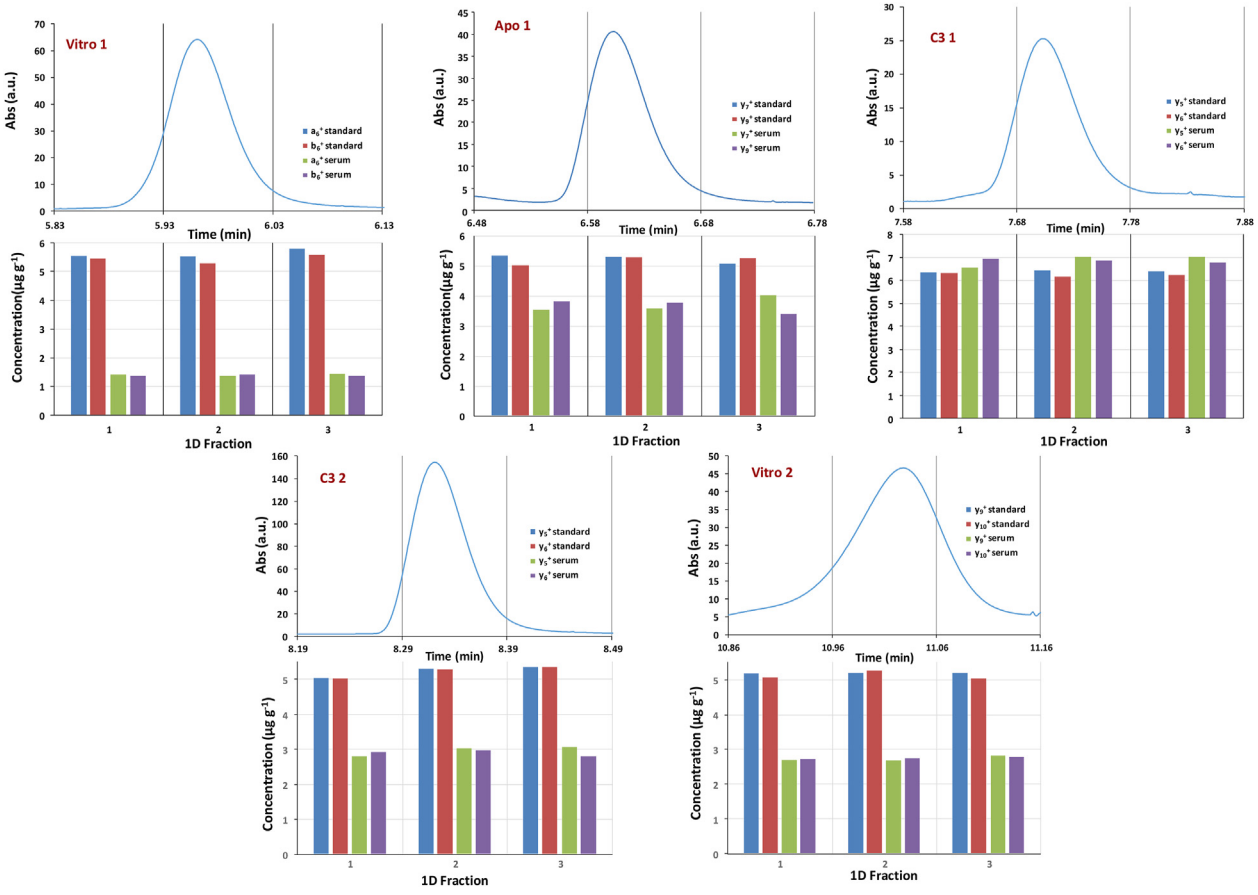

Fig. 2. Chromatographic peak profiles for VTN 1, APO 1, C3 1 C3 2 and VTN 2, time window of 40 μL fractions transferred to the second dimension and the concentrations obtained in each fraction for a standard mixture containing approximately 5 μg g^{-1} of each peptide and a pooled serum sample analyzed by high resolution sampling and IDMS using coeluting ^{13}C minimally labelled peptides.

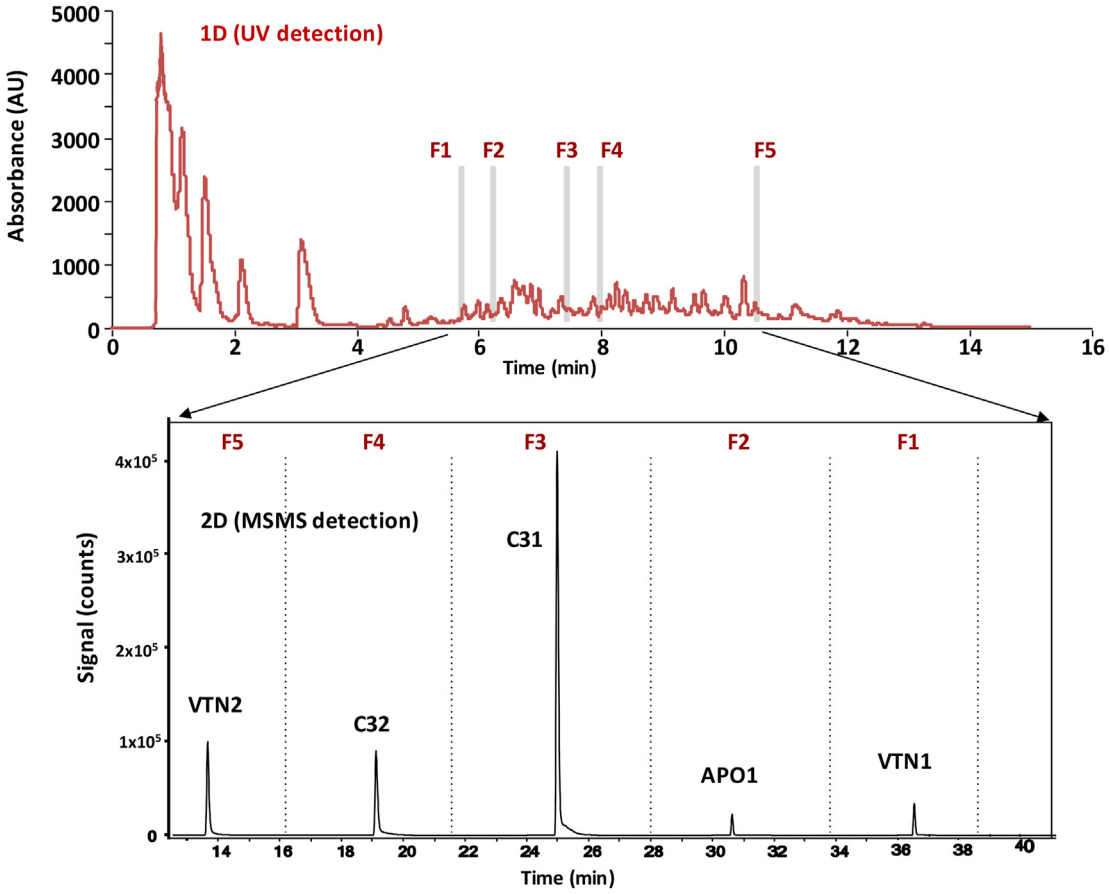

Fig. 3. 1DLC-UV and 2D-LC-ESI-MS/MS chromatograms of a pooled serum sample analyzed under the optimized conditions by IDMS using ^{13}C minimally labelled peptides. The grey lines indicate the time window of the fractions transferred to the second dimension.

recovery values from 96 to 102% were obtained for all fragments and peptides. The precision obtained between three independent injections by 2D-LC-MSMS ranged from 0.75% to 4.4% expressed as RSD (%) whereas the precision obtained between three independent replicates of the same sample ranged from 2.3 to 3.5% (%RSD). For VTN peptides recovery values ranged from 96 to 101% and precision between three independent injections by 2D-LC-MS/MS from 0.8% to 5% expressed as RSD (%). The precision obtained between three independent replicates of the same sample ranged for all fragments and peptides of VTN from 0.8 to 2% (%RSD). Finally, recovery values from 100 to 116% were obtained for C3 peptides with a precision between 2D-LC-MS/MS injections from 0.4 to 5.6% and from 1.2 to 2.6% between three independent replicates. The installation of a diverter valve and a T-piece with connection to the MS and to the diverter valve before the entrance of the mass spectrometer would avoid the introduction of salts from the 1D mobile phase and hence, would allow a higher number of consecutive sample injections into the ESI-MSMS system.

3.5. Analysis of human serum samples

Finally, the methodology was applied to the analysis of serum samples of n = 94 control subjects and n = 91 patients diagnosed with POAG. A volume of 0.05 mL of a 20 μg g^{-1} solution containing all labelled peptides was added to each serum sample and were further submitted to the sample preparation procedure described in the Experimental section. Table S7 shows the average concentration of APOA4, VTN and C3 obtained with two fragment ions of each of the proteotypic peptides (APO 1, VTN 1, VTN 2, C3 1 and C3 2) for both groups of serum samples. Fig. 4 shows the comparison of the results obtained for each fragment ion and each peptide in both groups. Error bars of Fig. 4 correspond to the standard deviation of n = 94 and n = 91 for the control and the patients diagnosed with POAG, respectively and show mainly the biological variability of the measurements. No statistical difference was found in the protein concentration values obtained not only between different fragment ions of the same peptide but also between different peptides of the same protein. Also, no statistical differences were found in the protein concentrations obtained when comparing both groups of samples (POAG vs controls, $p > 0.05$ for C3, VTN or APOA4, Mann Whitney *U* test with SPSS software v25.0 IBM Corp., NY, USA). According to our results, the validity of those proteins as potential POAG biomarkers previously demonstrated by immunoassays [33] is questionable. The proposed methodology demonstrated a satisfactory sample throughput as we were able to analyze 30 samples every five working days. Source cleaning was required after 30 injections of serum sample into the 2D-LC-ESI-MS/MS.

4. Conclusions

Automated multiple heart cutting combined with isotope dilution tandem mass spectrometry has been applied here as a peptide enrichment and an absolute quantitative strategy for the direct

Table 2
Concentration (μg g^{-1}) and recovery values (%) obtained in the analysis of serum samples fortified with known amounts of peptides APO 1, VTN 1 y VTN 2, C3 1 and C3 2 measuring the isotopic distribution of two different molecular fragment ions for each peptide by 2D-LC-MS/MS. Uncertainty values correspond to the standard deviation of n = 3 independent replicates.

Peptide	Ion Fragment	Replicate	Theoretical concentration (μg g^{-1})	Experimental concentration (μg g^{-1})	Recovery
APO 1	$\mathbf{y_7^+}$	1	31,18	30,2 ± 0,7	96,9 ± 2,1
		2	28,68	27,9 ± 1,4	97,2 ± 5,0
		3	28,87	29,2 ± 0,8	101,1 ± 2,9
		Average		**29,1 ± 0,4**	**98,4 ± 2,3**
	$\mathbf{y_9^+}$	1	30,75	29,5 ± 0,3	95,9 ± 1,1
		2	28,25	27,1 ± 0,2	96,1 ± 0,7
		3	28,44	29,0 ± 1,3	102,1 ± 4,5
		Average		**28,6 ± 1,2**	**98,0 ± 3,5**
VTN 1	$\mathbf{b_6^+}$	1	31,89	31,2 ± 1,5	97,9 ± 4,8
		2	29,37	28,2 ± 1,0	96,0 ± 3,4
		3	29,56	29,6 ± 0,4	100,1 ± 1,5
		Average		**29,7 ± 1,5**	**98 ± 2,0**
	$\mathbf{a_6^+}$	1	31,87	31,9 ± 1,2	100,0 ± 3,9
		2	29,36	28,6 ± 0,9	97,4 ± 3,1
		3	29,55	28,3 ± 1,1	95,9 ± 3,9
		Average		**29,6 ± 2,0**	**97,8 ± 2,1**
VTN 2	$\mathbf{y_{10}^+}$	1	34,86	34,9 ± 0,8	100,2 ± 2,4
		2	32,37	32,1 ± 0,3	99,2 ± 1,1
		3	32,56	32,7 ± 0,5	100,6 ± 1,6
		Average		**33,3 ± 1,5**	**100,0 ± 0,7**
	$\mathbf{y_9^+}$	1	34,84	34,6 ± 0,7	99,3 ± 2,1
		2	32,35	31,7 ± 0,2	98,0 ± 0,8
		3	32,54	33,0 ± 0,6	101,4 ± 2,0
		Average		**33,1 ± 1,4**	**99,6 ± 1,7**
C3 1	$\mathbf{y_6^+}$	1	36,20	37,5 ± 0,1	103,6 ± 0,4
		2	33,66	35,7 ± 0,7	106,0 ± 2,2
		3	33.85	35,3 ± 0,3	104,2 ± 0,8
		Average		**36,1 ± 1,2**	**105,6 ± 1,3**
	$\mathbf{y_5^+}$	1	35,97	38,0 ± 1,3	105,7 ± 3,8
		2	33,43	34,1 ± 0,2	102,0 ± 0,7
		3	33,63	34,7 ± 0,5	103,1 ± 1,4
		Average		**35,6 ± 0,6**	**103,6 ± 1,9**
C3-2	$\mathbf{y_5^+}$	1	36,86	40,8 ± 2,3	110,7 ± 6,2
		2	34,27	38,3 ± 1,9	111,8 ± 5,5
		3	34,46	40,1 ± 1,6	116,3 ± 4,6
		Average		**39,7 ± 0,3**	**113,0 ± 3,0**
	$\mathbf{y_6^+}$	1	38,63	39,5 ± 0,4	102,3 ± 1,0
		2	36,04	36,0 ± 1,0	100,0 ± 2,8
		3	36,23	37,0 ± 1,0	102,3 ± 2,9
		Average		**35,7 ± 1,8**	**101,5 ± 1,3**

analysis of tryptic digests of serum samples. Using this strategy, the sample preparation required, besides enzymatic digestion, is a simple centrifugation of the supernatant, evaporation and reconstitution in the 1D mobile phase. The most important benefit of using 2DLC instead of 1DLC is the minimization of matrix effects in the ESI source. This allowed us to avoid time-consuming sample purification steps and hence, increase sample throughput in comparison with previous works [36]. The methodology allows the use of mobile phases not compatible with the ESI source which provides improvements in chromatographic resolution of the target peptides and better pH stability. We have also demonstrated that potential bias arising from slight retention time shifts typically occurring in the analysis of serum samples and affecting the amount of analyte transferred to the second dimension is corrected using minimally ^{13}C labelled analogues that coelute with the target peptides. Satisfactory results were obtained when analyzing fortified serum samples (recoveries from 98 to 113%). In contrast to previous studies using immunoassays [33], the analysis of serum samples from control subjects and patients with POAG did not show any statistical difference in the APOA4, VTN and C3 concentrations. The methodology can be considered universal as it can be extended to a higher number of proteins as long as proteotypic peptides are chromatographically resolved in the first dimension and a coeluting labelled analogue for each peptide is added at the beginning of the sample preparation procedure.

CRediT authorship contribution statement

Amanda Suárez Fernández: performed all the analytical measurements. **Pablo Rodríguez-González:** wrote the manuscript, supervised the work, and acquired, Funding acquisition. **Lydia Álvarez:** carried out the patient selection, sample collection and preparation. **Montserrat García:** carried out the patient selection, sample collection and preparation. **Héctor González Iglesias:** designed the sample collection and acquired, Funding acquisition, for the project. **J. Ignacio García Alonso:** supervised the work and acquired, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have

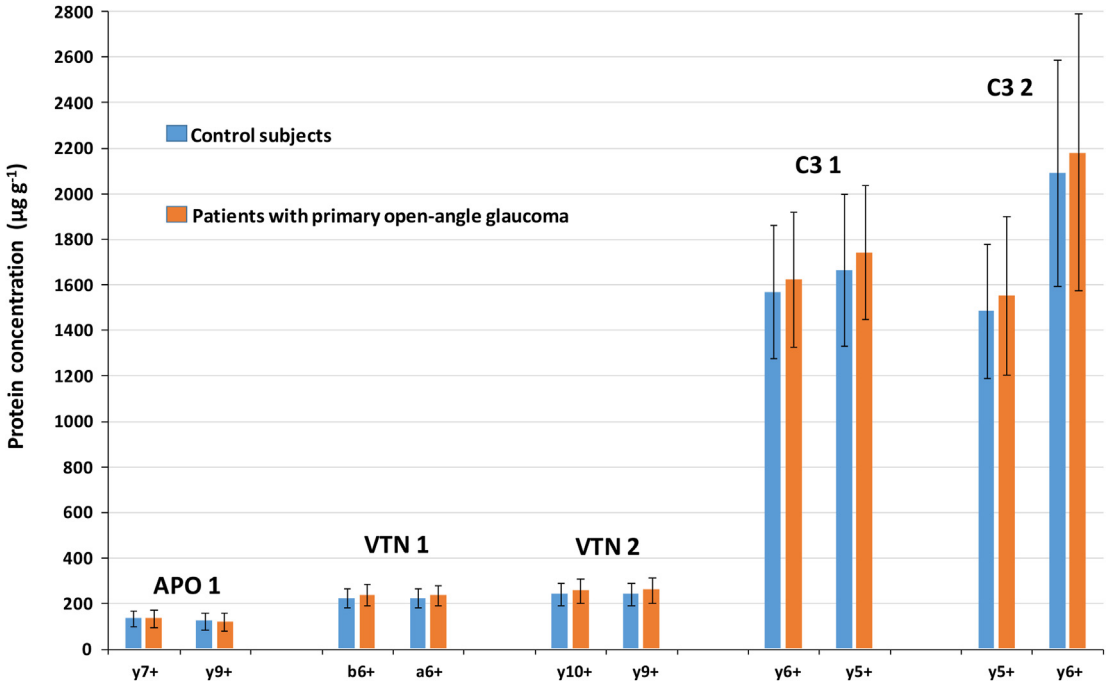

Fig. 4. Average concentration of APO, VTN and C3 in serum samples from n = 95 control subjects and n = 95 patients with primary open-angle glaucoma (POAG). Concentrations were obtained with two fragment ions of each of the proteotypic peptides APO 1, VTN 1, VTN 2, C3 1 and C3 2. Error bars correspond to the standard deviation of n = 95 individuals.

appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aca.2021.339022.

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